# A TECHNIQUE FOR THE ESTABLISHMENT OF MYCORRHIZAL INFECTION IN ORCHID TISSUE GROWN IN ASEPTIC CULTURE

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## SUMMARY

Two techniques for producing large quantities of mycorrhizal and non-mycorrhizal orchid tissue for experimental purposes are described.

Protocorm-like masses of *Dactylorhiza purpurella* (T. & T.A. Steph.) Soó, were grown axenically in darkness on 2% sucrose mineral salts medium with growth supplements at 22 °C. These cultures, derived from seed, were substantially larger than the original protocorms.

Mycorrhizal infection of this tissue was achieved successfully in 100 ml conical flasks in which the protocorm-like tissue was suspended on water agar only and the fungus inoculated on an adjoining cellulose, mineral salts slope.

Infection, using several *Rhizoctonia* strains in this double-culture system gave consistent mycorrhizas characteristic of natural protocorms and with extensive colonization of cortical tissue. Alternative inoculation methods using a single aqueous or agar medium and with recognized mycorrhizal strains, gave variable infection, frequently resulting in pathogenicity and host death. The advantage of the double culture system in providing large quantities of orchid mycorrhizal tissue for experimental studies is discussed.

## INTRODUCTION

Propagation of orchids by tissue culture is practised commercially for the clonal production of outstanding varieties. It also provides a valuable tool for research into the mycorrhizal relationship between orchid root and endophyte. Cultures have been derived from orchid explants, including leaf tips (Churchill, Ball and Arditti, 1973), shoot meristems (Marston, 1967) and from seeds, either by germination in the presence of the endophyte (Downie, 1959) or by non-mycorrhizal growth from germination on a high nutrient medium (Knudson, 1922). The maintenance of non-mycorrhizal cultures necessitates a delicate balance of organic nutrients, inorganic nutrients, growth factors (Hadley, 1970a; Hadley and Harvais, 1968; Harvais, 1972) and environmental conditions such as light and temperature (Harvais and Hadley, 1967). Mycorrhizal establishment, however, requires a different nutrient balance with lower sugar levels or a cellulose carbon source (Hadley, 1969). While low temperature (11 °C) was suitable for mycorrhizal growth (Harvais and Hadley, 1967) asymbiotic cultures grew poorly at this temperature (Hadley, 1970a).

Seed-derived protocorms provide only a limited amount of tissue for experimental work, and for this reason biochemical studies on orchid mycorrhizas have been largely cytochemical. The development of plantlets from these protocorms similarly

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gives only a small amount of root tissue available for infection. This paper describes the aseptic production of undifferentiated protocorm-like tissue and a technique designed to overcome the foregoing limitations and provide substantial quantities of orchid mycorrhizal tissue comparable to a natural infected root.

## MATERIALS AND METHODS

### *Culture of orchid and fungus*

Orchid cultures were derived from protocorms of *Dactylorhiza purpurella* (T. & T.A. Steph.) Soó and grown in 10 ml of orchid culture medium sterilized by millipore filtration (0·22 $\mu$m pore size) in 100 ml Erlenmeyer flasks and incubated at 22 °C in darkness. All fungal strains used were *Rhizoctonia* spp., which have been shown to produce the following perfect stages:

*Ceratobasidium* spp., T, Thrl, Rgr and W 57;
*Thanetephorus cucumeris*, Rsl, W 48, W 82 and W 87.
*Rhizoctonia repens* strain 0689 (probable perfect state *Tulasnella calospora*, J. Warcup pers. comm.).

Strains were maintained on PDA (Difco) at 22 °C and subcultured at 14-day intervals, or grown as aqueous cultures in Richard's medium (g $l^{-1}$) $KNO_3$, 10; $KH_2PO_4$, 5·0; $MgSO_4 . 7H_2O$, 0·25; $FeCl_3 . 6H_2O$, 0·035; potato starch, 10·0; sucrose, 50·0. Cultures were shaken on a gyratory shaker at 150 r $min^{-1}$ for 10 days in darkness.

The orchid culture medium (OCM) used for routine propagation of host tissue consisted of: *macronutrients* – (g $l^{-1}$) $NH_4NO_3$, 0·213; $(NH_4)_2SO_4$, 0·37; $KH_2PO_4$, 0·55; $KNO_3 . 4H_2O$, 1·05; $Ca(NO_3)_2 . 4H_2O$, 0·80; $MgSO_4 . 7H_2O$, 0·50; sucrose, 20·0: *micronutrients* – (mg $l^{-1}$) $CuSO_4 . 5H_2O$, 0·025; $H_3BO_3$, 6·2; KI, 0·83; $Na_2MoO_4 . 2H_2O$, 0·25; $MnSO_4 . 4H_2O$, 22·3; $ZnSO_4 . 7H_2O$, 8·6; $CaCl_2 . 6H_2O$, 0·025; Glycine, 2·0; *meso*-inositol, 100·0; nicotinic acid, 0·5; thiamine HCl, 0·5; pyridoxine HCl, 0·5; Fe Na EDTA, 25·3; kinetin, 0·2; IAA, 2·5. Other media used were modifications of Pfeffer's medium, with the following additions: 1 % glucose (1 % Pf) (Hadley, 1969, 1970a); 0·1 % glucose (0·1 % Pf) (Hadley, 1969, 1970b) and 2 % cellulose (Merck non-ball-milled microgranular) (2 % CPf) (Williamson, 1973).

### *Inoculation*

Orchid cultures on liquid media were established by transferring an entire protocorm mass, or a cut portion of one, to the flask. Cultures on agar media were prepared by partially embedding the protocorm-like tissue into macerated agar to prevent desiccation.

The fungal strains as used in the imperfect stage were all sterile mycelia and lacked the spore-like bodies described by Curtis (1939). Inoculation was either by 0·5 cm discs of agar and mycelium, or a suspension of mycelial fragments. *Rhizoctonia* cultures for disc inoculum were prepared from PDA plates inoculated with a central disc of mycelium and incubated for 10 to 14 days in darkness. Mycelial fragments were prepared from 2-week-old aqueous cultures in Richard's medium, washed twice in 0·1 M pH 5·2 acetate buffer, centrifuged at 1400 *g* and homogenized in the proportions 1:5 (w/v) mycelium:buffer, in a Sorvall homogenizer at 5000 r $min^{-1}$ for 3 min. At speeds and times exceeding this, viability was greatly reduced. An alternative method of preparing mycelial fragments was

to remove surface growth from PDA cultures in 10 ml acetate buffer by gentle abrasion with an inoculating loop. All inoculations and transfers were carried out in a sterile, laminar-airflow cabinet.

*Measurements of orchid growth*

Fresh wts of infected and non-infected tissue were determined, after washing and blotting dry. Growth was assessed as the incremental fresh wt increase (W) per gram of initial tissue inoculum. The initial weight ($W_0$) was obtained by subtraction of predetermined weights of flask and medium from that of the freshly inoculated cultures. Growth was also measured as dry wt of individual cultures after 48 h at 70 °C. Growth of the orchid explant on plain agar in the absence of carbon or nutrients was evidence of mycorrhizal establishment together with the appearance of hyphal pelotons in tissue sections examined microscopically. No growth occurred on this medium in the absence of the fungus. Freehand sections were examined without staining. Paraffin-embedded tissue was dehydrated through a tertiary butyl alcohol series and sections stained in Delafield's haematoxylin for 20 min, or by the periodic acid Schiff's (P.A.S) reaction.

## Results

*Growth of orchid protocorm in the absence of the mycorrhizal symbiont*

Growth of axenic protocorm cultures in darkness occurred only on a medium containing a carbon source. The undifferentiated growth of protocorms after 4 weeks on OCM, 1 % Pf, 0·1 % Pf and 2 % CPf over a temperature range of 20 to 28 °C is shown in Figure 1. At temperatures above 28 °C rapid dehydration of the medium occurred and below 15 °C growth was negligible. Maximal growth occurred on 1 % Pf at 26 °C, but this was unsatisfactory for long-term cultures

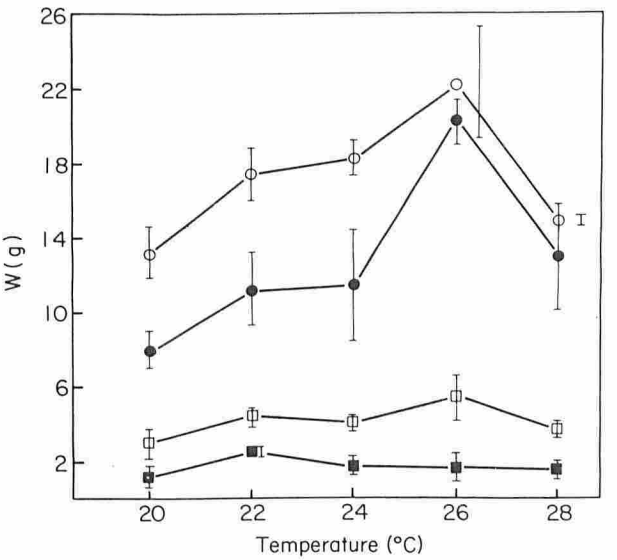


Fig. 1. Fresh wt increments (W) of axenic orchid cultures grown for 4 weeks on different media over a range of temperatures. ●, Orchid culture medium; ○, 1 % glucose, Pfeffer's medium; □, 0·1 % glucose, Pfeffer's medium; ■, 2 % cellulose Pfeffer's medium. Vertical bars represent the standard error of the mean of five replicates.

due to excessive browning of the tissue. Subsequent experiments using Murashige and Skoog's (1962) medium, which differed from OCM largely in the omission of $(NH_4)_2SO_4$, $CuSO_4$, $CaCl_2$ and $NaMoO_4$ and in a much higher molar strength, produced protocorms with similar morphology to those grown on OCM, but the growth rate was lower than on OCM which was used subsequently for routine production of axenic protocorms. Healthy, rapidly growing tissue assumed an elongated bulbous form with a translucent or opaque appearance. Additionally, long rhizoidal branches with epidermal hairs developed or occasionally, small spherical structures. Neither of these forms produced good growth when used as inocula.

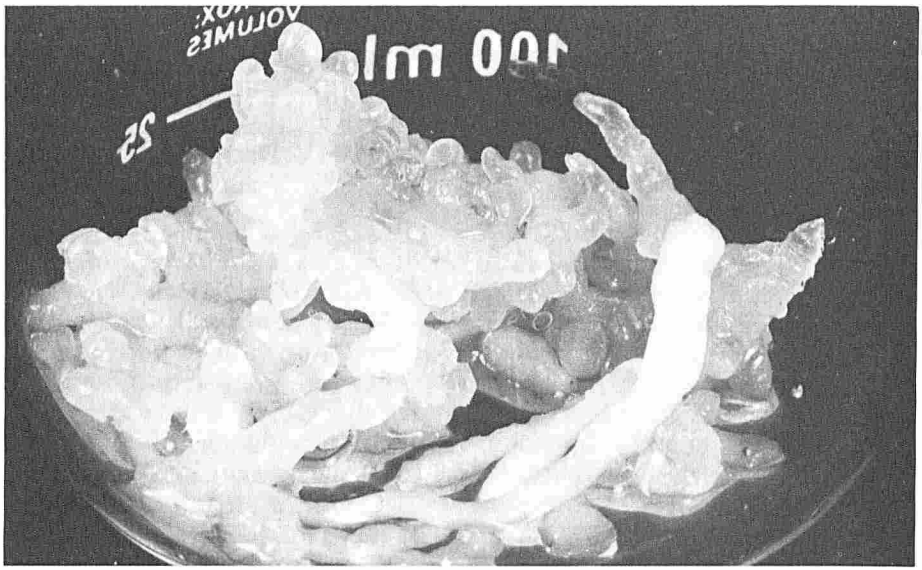

Fig. 2. Axenic growth of *Dactylorhiza purpurella* undifferentiated protocorm-like tissue on aqueous orchid culture medium (see text).

For routine axenic protocorm production six, 5 to 10 mm protocorm segments, detached at a branch point, were placed in 10 ml OCM in 100 ml wool-plugged conical flasks and incubated in darkness at 22 °C. The growth obtained after 12 weeks is shown in Figure 2. Cultures could be maintained for 16 weeks before depletion of the medium necessitated sub-culturing.

*Establishment of mycorrhizal growth*

Attempts to induce mycorrhizal growth by placing a strip of PDA and mycelium as inoculum adjacent to a cut surface of a protocorm partially embedded in 0·1 % Pf agar were unsuccessful. Daily observations up to 20 days after inoculation showed that the *Rhizoctonia* strains T, W 48 and W 82 all invaded pathogenically causing browning and disintegration of the protocorm. W 48 penetrated by infection cushions characteristic of the pathogenic *Rhizoctonia*, while strains T and W 82 invaded directly. When the fungus and protocorm were juxtaposed on a high nutrient medium such as OCM, or 1·0 % Pf, the protocorm was covered with excessive growth of the fungus and pathogenesis ensued even in the absence of a cut surface. Limited success was achieved in establishing

TABLE 1. *Mycorrhizal growth in* Dactylorhiza purpurella *protocorms in aqueous 2% cellulose Pfeffer's medium inoculated with* Rhizoctonia *strain T mycelial fragments*

| Time (days) | Dry wt of orchid protocorm (mg) (Means of 10 replicate cultures) Non-inoculated | T-inoculated | L.S.D. |
|---|---|---|---|
| 0 | 18·0 | 20·3 | 5·0 |
| 7 | 17·6 | 21·3 | 6·1 |
| 14 | 16·9 | 15·4 | 6·8 |
| 21 | 19·1 | 26·9 | 6·9* |
| 28 | 16·2 | 31·8 | 13·6*** |

*, ***, $P = 0{\cdot}05$ and 0·001 respectively.

mycorrhizal growth in protocorm cultures transferred to aqueous cellulose Pfeffer's medium (2% CPf) in which the carbohydrate source (cellulose) was unavailable to the host. Protocorm pieces of approximately 18 mg dry wt from OCM were transferred to 10 ml aqueous 2% CPf in 100 ml conical flasks and inoculated with a suspension of mycelial fragments of the T strain (see Methods). No increase in protocorm dry wt occurred in non-inoculated cultures (Table 1). Evidence of mycorrhizal-induced growth was based on the increased dry wt at 21 days after inoculation. After 28 days inoculated cultures showed a 96% increase in dry matter over the controls. This method was unsatisfactory as an experimental technique for routine production because of excessive fungal growth over the protocorms and the resulting high percentage of cultures which became pathogenic and rotted.

In view of the difficulties experienced with aqueous media, the double-culture technique shown in Figure 3 was devised. The media in 100 ml conical flasks

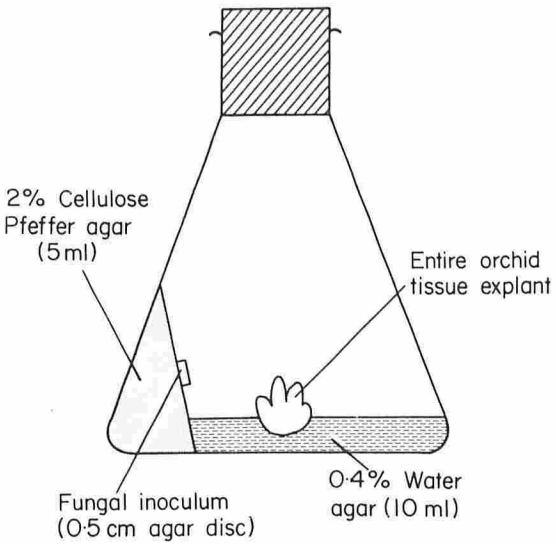


Fig. 3. Diagrammatic representation of a double-medium culture system used for the establishment of mycorrhizas on orchid tissue.

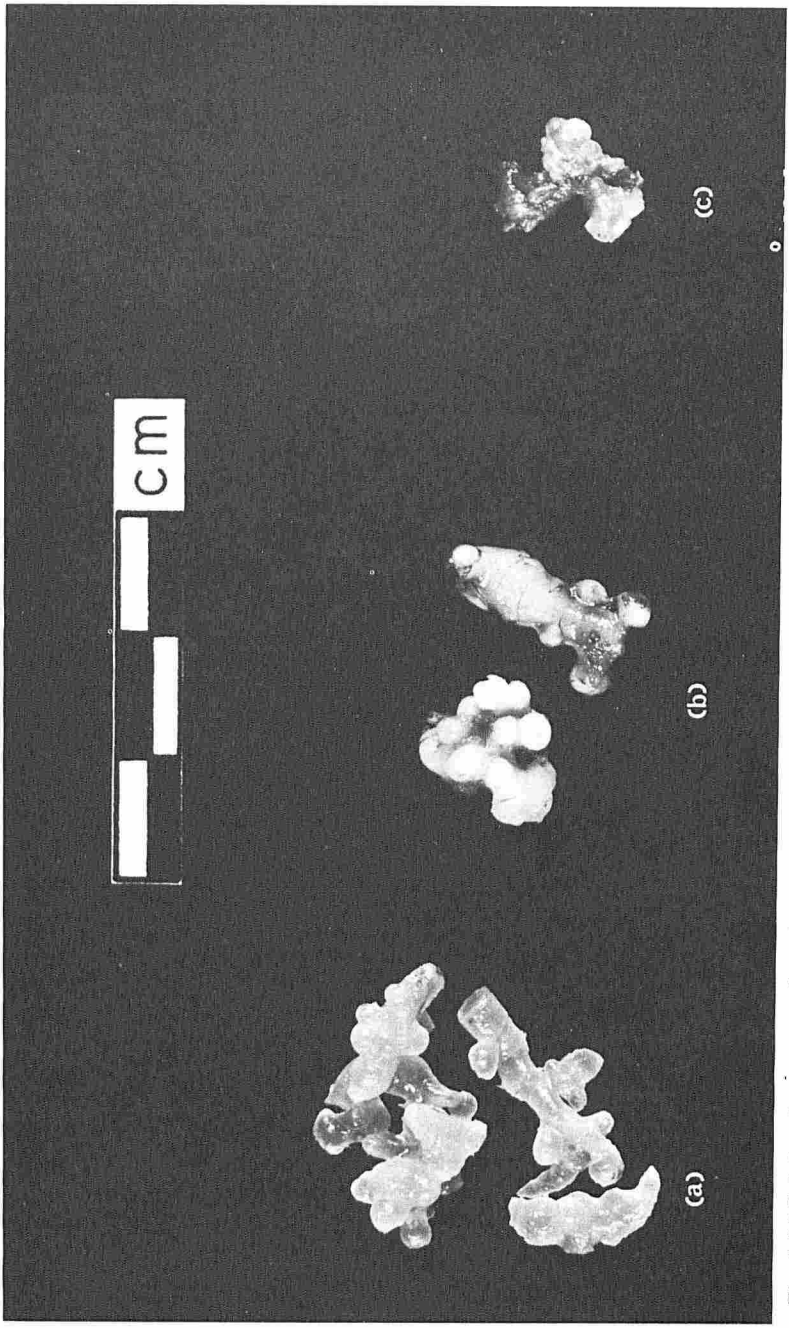


Fig. 4. (a) Uninfected protocorm pieces from a bulk culture grown on aqueous OCM; (b) mycorrhizal protocorm grown in double culture (see Fig. 2) 5 weeks after inoculation with *Rhizoctonia* strain T; (c) Pathogenic reaction in protocorm tissue induced by strain T inoculated on to an OCM agar protocorm culture.

Fig. 5. Section of mycorrhizal protocorm showing early to middle stage peloton formation in cortical parenchyma cells 14 days after inoculation with *Rhizoctonia* strain T.

consisted of a basal non-nutritive support medium (0·4 % water agar) for the protocorm and an adjoining near-vertical slope of 2 % CPf agar for fungal growth. Orchid protocorms (approximately 18 mg dry wt) were transferred from OCM stock cultures and partially embedded in the water agar. The 2 % CPf slope was inoculated with a 0·5 mm disc of PDA and mycelium of the appropriate fungal strain. Mycelial growth was sparse over the non-nutritive agar, resulting in a minimum of external growth over the protocorm.

Figure 4 illustrates mycorrhizal-induced growth in the double-culture 5 weeks after inoculation with strain T. Uninfected protocorms (A) are pieces obtained from a 12-week-old OCM bulk culture. Tissue (C) is a pathogenic reaction induced by T inoculated into an OCM agar culture of the protocorm.

Infection occurred at between 14 and 21 days, resulting in dense peloton formation in the cortical tissue in the lower two-thirds of the protocorm-like mass (Fig. 5). Good growth was maintained for 10 weeks after inoculation. Mycorrhizal cultures were also successfully established with strain 0689, and less extensively with strains W 57 and Rgr at 22 °C, but only with 0689 at 10 °C. Strains W 82 and Thrl showed little tissue penetration whereas W 48, W 87 and Rsl proved to be highly pathogenic at 22 °C.

## DISCUSSION

Studies on the growth of axenic orchid tissue confirmed the observations of Harvais and Hadley (1967) that non-mycorrhizal growth is restricted on low carbon sources, represented in the present work by 0·1 % Pf and 2 % CPf. The orchid culture medium gave the most balanced growth in the absence of the fungus and was subsequently used successfully for the maintenance and bulking of large quantities of orchid tissue for mycorrhizal studies. These cultures were high in starch reserves in the uninfected state, as seen in seed-derived asymbiotic protocorms of other orchids (Purves and Hadley, 1976), but starch was rapidly lost on mycorrhizal establishment. Maximal growth occurred at 26 °C, but led to more rapid dehydration of the cultures. Routine cultures were produced in the range 22 to 24 °C.

The double-culture system gave consistent and extensive mycorrhizal infection. This was readily detected as new growth, since the only source of nutrients available to the host was that translocated from the fungal symbiont after infection. Pathogenic infection characterized by brown lesions and degradation of host tissue readily distinguished this from the mycorrhizal tissue. The sparse mycelial overgrowth of the tissue permitted a ready separation of non-infecting fungus from mycorrhizas at harvest. The orchid tissue culture referred to here is not *sensu stricto* a protocorm as that developed from a germinating seed, however, the tissue retained the essential characteristics of the seed-derived structure, exhibiting uninfected epidermal cells, central vascular tissue and peloton formation restricted to the cortical parenchyma. Mycorrhizal growth readily established on mature tissue by the double-culture method. On transfer to a 15-h photoperiod these cultures differentiated into typical orchid plantlets. Finally, light microscopy showed comparable cytology to other mycorrhizal infections (Hadley, 1975). It would thus appear that this tissue is analogous to naturally grown protocorms, and results may be extrapolated to orchid mycorrhizal studies in general.

Excessive fungal growth, characterized by infection on single high-sugar media, promoted pathogenesis as did invasion through a cut surface.

The extreme variation found in infection types resulting from different inoculation procedures, incubation temperatures and media, emphasizes the fine balance existing between the pathogenic and mycorrhizal states, and the importance of medium composition and inoculum potential in mycorrhizal studies. The main advantage of the double-culture is that it enables the study of mycorrhizas in experiments requiring much larger quantities of tissue than are readily available from seed-derived protocorms (Williamson and Hadley, 1970). It also makes possible infectivity experiments with known fungal strains on mature tissue under fully defined, aseptic conditions, which are not applicable to mature, non-sterile orchid root systems. Biochemical and histochemical investigations of the host–parasite relationship using the mycorrhizal double-culture system are currently in progress and will be reported later.

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